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Preservation of FoxP3$^+$ Regulatory T Cells in the Peripheral Blood of Human Immunodeficiency Virus Type 1-Infected Elite Suppressors Correlates with Low CD4$^+$ T-Cell Activation$^\nabla$

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Elite suppressors (ES) are untreated human immunodeficiency virus type 1 (HIV-1)-infected individuals who maintain normal CD4$^+$ T-cell counts and control viremia to levels that are below the limit of detection of current assays. The mechanisms involved in long-term control of viremia have not been fully elucidated. CD4$^+$ CD25$^+$ regulatory T cells (Tregs) downmodulate chronic inflammation by suppressing the activation and proliferation of effector lymphocytes. We found that while Tregs were functional in ES and patients on highly active antiretroviral therapy (HAART), ES maintained high levels of Tregs in peripheral blood mononuclear cells whereas patients on HAART had evidence of Treg depletion. We also demonstrated that Tregs can serve as reservoirs for HIV-1 in vivo. These data suggest that both direct infection by HIV-1 and tissue redistribution are possible explanations for declining FoxP3$^+$ Tregs in progressive HIV-1 infection. Furthermore, the maintenance of Tregs may be one mechanism associated with the nonprogressive nature of HIV-1 infection in ES.

CD4$^+$ CD25$^+$ regulatory T cells (Tregs) are a unique population of T cells that suppress the activation and proliferation of effector lymphocytes (33, 35, 46, 49, 51–53, 55). Tregs specifically and exclusively express FOXP3, a transcription factor that plays a key role in their development and function (22, 29–30). Ligation of CD80 or CD86 on effector cells by the cell surface molecule CTLA-4 on Tregs results in suppression of the effector cells (46). Suppression of effector cells by Tregs can also be mediated over a short range by cytokines (interleukin 10 [IL-10] and transforming growth factor β), by direct cell contact, or by mechanisms that "instruct" antigen-presenting cells (APCs) to increase tryptophan metabolism to interfere with T-cell activation (55). The role of Tregs in human immunodeficiency virus type 1 (HIV-1) pathogenesis remains unclear. One hypothesis holds that these cells prevent chronic immune activation and are therefore beneficial. Alternatively, it is possible that these cells suppress the antiviral immune response and are therefore harmful.

Several studies have shown that despite elevated CD25 expression on CD4$^+$ T cells, there is a depletion of Tregs from peripheral blood mononuclear cells (PBMCs) in patients with progressive HIV-1 disease. These and other data have led some investigators to conclude that Tregs play a beneficial role by limiting the apoptosis of uninfected CD4$^+$ T cells that results from high levels of chronic immune activation (6, 8, 15, 18, 32, 41, 44, 54). On the other hand, it is possible that Tregs blunt HIV-1-specific immunity, allowing for uncontrolled viral replication in lymphoid tissue. In support of this hypothesis, studies showing decreased Tregs in PBMCs and increased Tregs in lymphoid organs suggest that there may be a redistribution of these cells in patients with progressive HIV-1 infection (5, 11, 21, 31, 42).

Some HIV-1-infected individuals, termed long-term nonprogressors (LTNP), remain asymptomatic and maintain high CD4$^+$ T-cell counts for many years without antiretroviral treatment (14, 27, 45). A subset of LTNP, termed elite suppressors (ES), maintain viral loads of <50 copies of HIV-1 RNA/ml of plasma and normal CD4$^+$ T-cell counts without therapy (16, 38, 48). Understanding the mechanisms by which ES control viremia may help us determine the factors that affect the rate of disease progression in HIV-1-infected individuals (2, 4, 10, 19, 39, 56). Previous studies have shown that the relative absence of Tregs in the lymphoid tissue from simian immunodeficiency virus- and HIV-infected LTNP was associated with durable virologic control (11, 42).

HIV-1 causes a persistent infection characterized by immune activation and a progressive loss in the number and function of CD4$^+$ T cells. After treatment with highly active antiretroviral therapy (HAART), CD4$^+$ T-cell numbers recover but their function remains persistently suppressed (12, 34). Hence, progressive and nonprogressive HIV-1 disease represents a range of immunological scenarios with potentially different outcomes for Tregs (26). In addition, it has been shown that Tregs are highly susceptible to productive HIV-1 infection in vitro (28, 44). We sought to determine the frequency, functionality, and rate of infection of Tregs in the PBMCs of ES in comparison to patients on HAART.

MATERIALS AND METHODS

Human subjects. Healthy subjects (n = 8) were adults who were HIV-1 negative. Three groups of HIV-1-seropositive patients were included in the study: asymptomatic patients who maintained viral loads of <50 copies/ml without antiretroviral therapy (ES; n = 12), asymptomatic patients who had achieved

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TABLE 1. Clinical characteristics of study participants[a]

HIV-positive group or patient	CD4 count (cells/mm^3)	Amt of plasma HIV RNA (copies/ml)	Gender[b] (no. [%])	Age [yr]	Date [yr] of diagnosis
Chronic progressors					
2	325	91,698	M	46	2003
13	618	5,245	M	30	2003
34	774	<400	M	49	2002
37	754	47,486	M	22	2005
38	481	14,633	M	56	1999
39	369	3,740	M	47	2005
41	527	8,285	F	37	2002
HAART-treated patients	638 (477–683)[c]	<50	M (14 [67]), F (7 [33])	46 (33–63)[d]	1995 (1984–2006)[d]
ES					
ES2	683	<50	F	52	1986
ES4	837	<50	M	51	1996
ES3	775	<50	F	56	1991
ES5	831	<50	F	57	1990
ES6	965	<50	F	51	1992
ES7	1323	<50	M	53	1994
ES8	607	<50	M	56	2003
ES9	732	<50	F	57	1999
ES10	1160	<50	F	38	2002
ES11	781	<50	F	65	1993
ES14	939	<50	M	52	2002
ES19	952	<50	M	57	1996

[a] Clinical characteristics at time of blood draw.
[b] F, female; M, male.
[c] Median (interquartile range).
[d] Median (range).

suppression of viremia to <50 copies/ml on a stable HAART regimen ($n = 21$), and viremic patients ($n = 7$). Inclusion criteria for aviremic HIV-1$^+$ patients were the following: (i) on HAART for more than 1 year, (ii) suppression of viremia to <50 copies/ml for more than 1 year, and (iii) CD4 count of >200 cells/μl (in order to ensure that enough CD4$^+$ T cells could be isolated for experiments). Inclusion criteria for viremic HIV-1$^+$ patients were the following: (i) lack of prior antiretroviral therapy or lack of antiretroviral therapy for more than 1 year prior to study entry and (ii) viremia of >1,000 copies/ml for more than 1 year. An exclusion criteria for all blood donors was an AIDS-defining illness within 1 year of blood draw. Table 1 lists the pertinent clinical characteristics of the patients studied. All subjects provided written consent, and the protocol was approved by the Institutional Review Board of Johns Hopkins University School of Medicine.

Isolation of CD4$^+$ T cells from peripheral blood. PBMCs were isolated by density gradient centrifugation. For flow cytometry analysis and real-time reverse transcription-PCR (RT-PCR), CD4$^+$ T lymphocytes were isolated via positive selection with human CD4 MicroBeads (Miltenyi). For quantitative PCR (qPCR), suppression, and proliferation assays, PBMCs were first negatively selected to remove CD8$^+$ T cells, B cells, monocytes, and NK cells using mouse monoclonal antibodies to appropriate cell surface markers and magnetic beads conjugated with antibodies to mouse immunoglobulin G (Becton-Dickinson and Dynal Biotech). Further purification of Tregs was accomplished by sorting on a MoFlo cell sorter (DakoCytomation).

FOXP3 analysis. RNA was extracted from 2×10^6 peripheral blood CD4$^+$ T cells with RNAEasy (Qiagen), and 300 ng of RNA was reverse transcribed using random hexamers and the SuperScript III First-Strand synthesis system (Invitrogen). Quantitative real-time RT-PCR was carried out using the Applied Biosystems 7300 real-time PCR system. Standard curves were generated by cloning FOXP3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR products into plasmids using Platinum Pfx and the Zero Blunt TOPO PCR cloning kit (Invitrogen). The following primers were used: for GAPDH, 5′-GGAAGGTG AAGGTCGGAGTCAACG-3′ (sense) and 5′-CTGTTGTCATACTTCTCATG GTTCAC-3′ (antisense); for FOXP3, 5′-GCACCTTCCCAAATCCCAGT-3′ (sense) and 5′-GCAGGCAAGACAGTGGAAACC-3′ (antisense). In vitro transcription was performed using the MEGAscript T7 kit (Ambion). For the quantitation of FOXP3 gene expression, we used a commercially available kit (TaqMan Assay-on-Demand; Applied Biosystems). To normalize for RNA input, the sample content of GAPDH was quantified with the TaqMan reagents for GAPDH (Applied Biosystems).

Flow cytometry. For flow cytometric characterization of Tregs, CD4$^+$ T cells were stained with directly conjugated monoclonal antibodies specific for the CD4, CD25, CD62L, Ki67, CD69, CD45RO, CTLA-4, HLA-DR (BD Pharmingen), GITR, or FoxP3 (clone PCH101; eBioscience) protein or with appropriate isotype controls. Flow cytometric analysis was performed using the FACSCalibur system with CellQuest software (Becton Dickinson).

qPCR assay for HIV Gag DNA. Treg-cell-associated viral DNA was measured using a qPCR assay for HIV *gag* using the Applied Biosystems 7300 real-time PCR system. Gag primer sequences were as follows: gagF, GGT GCGAGAGCGTCAGTATTAAG; gagR, AGCTCCCTGCTTGCCCATA; the probe was gagP, FAM-AAAATTCGGTTAAGGCCAGGGGGAAA GAA-BHQ (Biosearch Technologies). CD4$^+$ FoxP3$^+$ CD25$^+$ T cells and CD4$^+$ FoxP3$^-$ CD25$^-$ T cells (3×10^4) were sorted into wells of a 96-well plate and lysed in 30 μl of 200 μg/ml proteinase K (Roche). For viremic patients, fluorescence-activated cell sorter (FACS)-purified CD4$^+$ CD25^{hi} CD62L^{hi} Treg and CD4$^+$ CD25$^-$ resting T cells were isolated and lysed. qPCR was performed on 5 μl of cell lysate for 45 cycles using Platinum *Taq* (Invitrogen). Standards were constructed for absolute quantification of Gag from sequential dilutions of a cell line which contains one copy of Gag per cell. Triplicate reactions were run and template copies calculated using ABI 7300 software.

In vitro suppression assays. FACS-purified CD4$^+$ CD25$^-$ CD62L^{hi} T cells (1×10^5; responders) were incubated with 1 μM carboxyfluorescein succinimidyl ester (CFSE) for 10 min at room temperature, washed thoroughly, and then stimulated for 6 days with 1×10^5 irradiated (3,000 rad) autologous PBMCs as APCs and 0.5 μg/ml anti-CD3 antibody in 96-well round-bottomed plates (Corning). To measure suppressive activity, 3×10^4 FACS-purified CD4$^+$ CD25^{hi} CD62L^{hi} Tregs were added at day zero. On day 6 or 7, proliferation of responders was measured by CFSE dilution using a FACSCalibur system with CellQuest software (Becton Dickinson). Percent suppression was calculated as follows: (proliferation of CD4$^+$ CD25$^-$ CD62L^{hi} T cells in the presence of CD4$^+$ CD25^{hi} CD62L^{hi} T cells/proliferation of CD4$^+$ CD25$^-$ CD62L^{hi} T cells in the absence of CD4$^+$ CD25^{hi} CD62L^{hi} T cells) × 100. All suppression assays were set up in triplicate, and results were expressed as a mean of triplicates.

In vitro proliferation assays. To analyze Treg proliferative capacity, 3×10^4 FACS-purified $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs were added to a Transwell insert (24-well type, pore size 0.4 μm; Costar). PBMCs (2×10^6) were added to each bottom well in medium containing 0.5 μg/ml anti-CD3 antibody, 1 μg/ml anti-CD28 antibody, and 100 U/ml recombinant IL-2. Either 5 μg/ml of HIV-1 p24 (Protein Sciences) or 0.1 μg/ml staphylococcal enterotoxin B (Sigma) was added to bottom wells. Cells were incubated for 6 days, pulsed with [^{3}H]thymidine for the final 16 h, and harvested. All proliferation assays were set up in triplicate, and results were expressed as a mean of triplicates.

Statistical analysis. The significance for all comparisons was calculated using Student's two-tailed *t* test assuming unequal variance. Spearman's rank test was used to determine the correlation between two variables. Statistical significance is defined as a *P* value of <0.05.

RESULTS

Inverse correlation between peripheral blood FOXP3$^+$ Treg levels and CD4$^+$ T-cell activation in patients with progressive HIV-1 disease. Previous studies have documented a decrease in the frequency of Tregs from the peripheral blood of patients with progressive disease (6, 8, 18, 44, 54). To determine the relative abundance of Tregs in PBMCs of ES, we used a real-time RT-PCR assay to quantify the expression of FOXP3, the most specific marker of Tregs (22, 29–30), in $CD4^+$ T cells. FoxP3 is important in distinguishing Tregs from activated/effector cells within the $CD25^+$ T-cell population, especially in settings of immune activation (23–24). The purity of $CD4^+$ T cells, positively selected from PBMCs, was consistently between 98 and 100% (data not shown). FOXP3 mRNA expression in the peripheral blood of aviremic HAART-treated and viremic patients was significantly decreased compared to levels in ES ($P < 0.001$) (Fig. 1A). The Treg copy number in the peripheral blood of patients with progressive disease was also decreased compared to that for uninfected patients ($P < 0.05$). Among all HIV-1-infected patients studied, those with higher CD4 counts tended to have higher FOXP3 copy numbers, but the trend did not reach statistical significance ($P = 0.06$) (Fig. 1B).

Consistent with the observed decrease in FOXP3 mRNA in PBMCs of patients on HAART, expression of the FoxP3 protein in $CD4^+$ T cells, as determined by intracellular staining, was significantly decreased compared to that for ES and uninfected patients ($P < 0.01$). For each patient, a pure population of $CD4^+$ T cells was surface stained with CD25-fluorescein isothiocyanate, permeabilized, and stained with FoxP3-phycoerythrin. Interestingly, for the patients on HAART, a majority of the $CD4^+$ T cells staining positive for FoxP3 came from the $CD25^+$ subset. However, for ES and uninfected patients, both the $CD25^-$ and $CD25^+$ subsets coexpressed FoxP3 (Fig. 2A). In mice, it has been shown that a majority of $CD4^+$ $FOXP3^+$ T cells have regulatory activity irrespective of their CD25 expression (7, 23, 43). Patients on HAART had significantly greater frequencies of activated $CD4^+$ T cells ($CD4^+$ $HLA\text{-}DR^+$) than was the case for ES and uninfected patients ($P < 0.01$). The percentage of $CD4^+$ T cells coexpressing CD25 was higher for HAART-treated patients than for ES patients ($P = 0.052$) (Fig. 2B, top graph). FoxP3$^+$ Tregs from the peripheral blood of all patient groups examined coexpressed similar levels of HLA-DR, CD69, and Ki67 (Fig. 2B, bottom graph). Importantly, the FOXP3 copy number was negatively correlated ($P < 0.05$) with the frequency of activated $CD4^+$ T cells, suggesting that Tregs might be important in controlling immune activation (Fig. 2C).

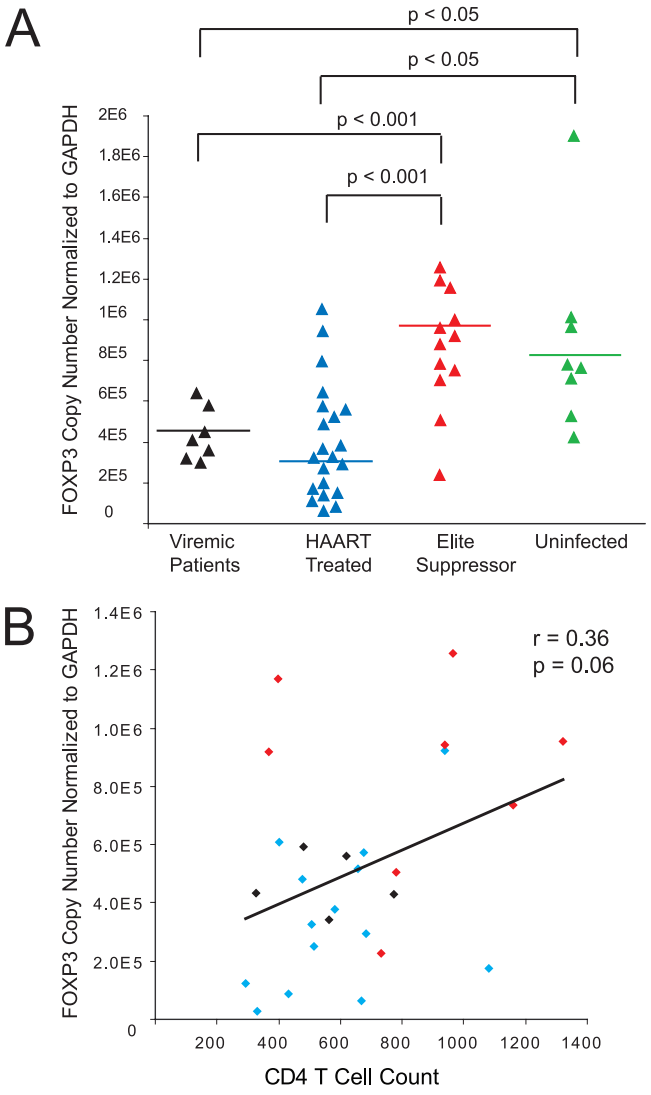

FIG. 1. Depletion of FOXP3$^+$ Tregs from progressive patients. (A) Real-time qRT-PCR of FOXP3 expression in peripheral blood $CD4^+$ T cells from treatment-naive (black), HAART-treated (blue), ES (red), or uninfected (green) patients. FOXP3 expression was significantly decreased in $CD4^+$ T cells from HAART-treated and treatment-naive patients compared to that for ES and uninfected patients. The copy number of FOXP3 in each sample was normalized to GAPDH. Horizontal lines represent the mean for each patient group. (B) Correlation between FOXP3 copy number in $CD4^+$ T cells from HIV-infected donors (treatment-naive [black], HAART-treated [blue], or ES [red]) and $CD4^+$ T-cell count. The *P* value indicates the level of significance.

Depletion of peripheral blood FoxP3$^+$ Tregs in HIV-1 infection can be partially explained by direct infection. We used qPCR to quantify the cell-associated virus in sorted FoxP3$^+$ Tregs in order to determine whether direct HIV-1 infection of Tregs could account for their depletion from the peripheral blood of patients with progressive disease. $CD4^+$ FoxP3$^+$ $CD25^+$ Tregs were infected at a mean frequency of 5.7 per 10^5 cells in patients on HAART and 0.4 per 10^5 cells in ES. This

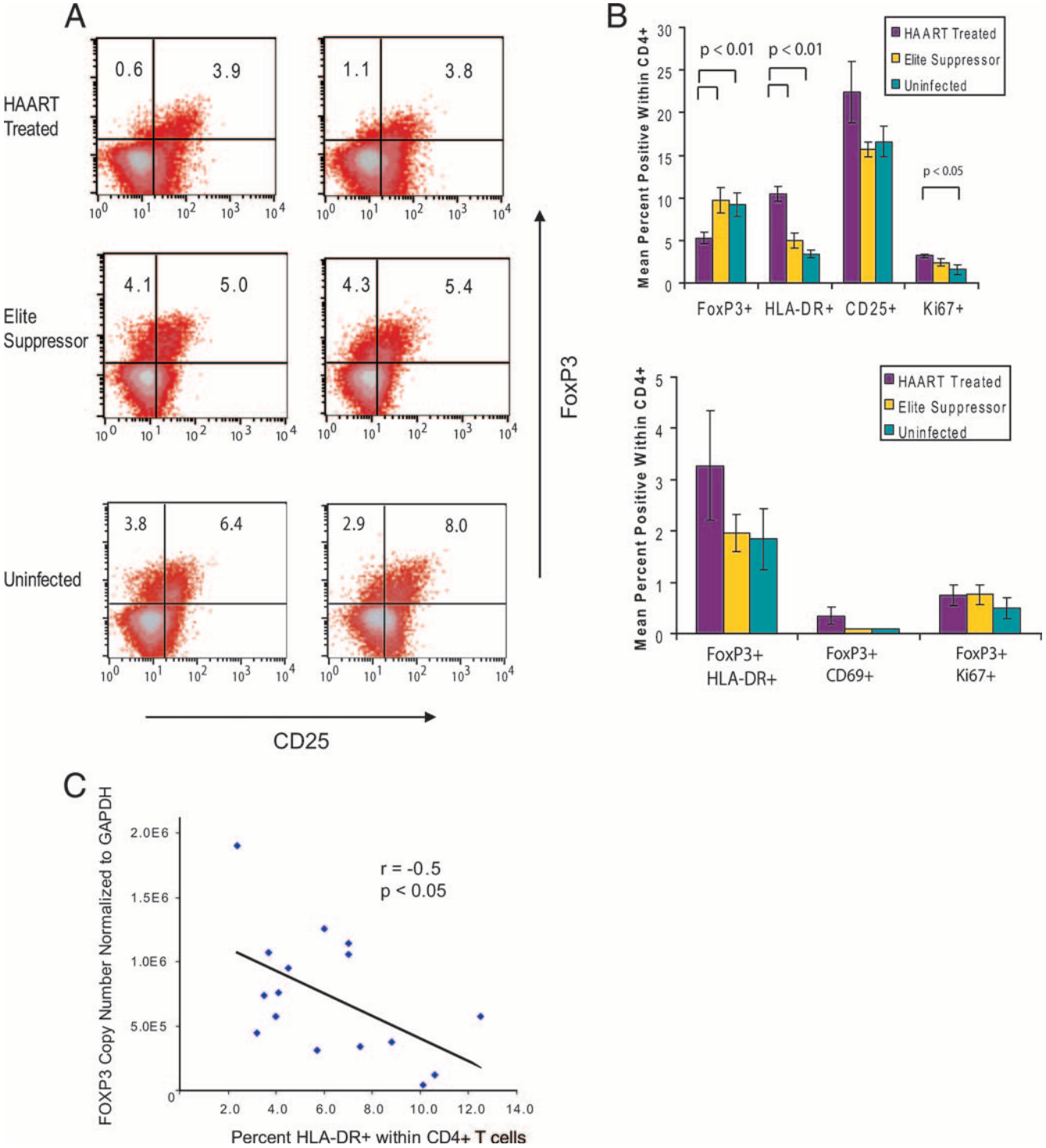

FIG. 2. Depletion of FoxP3-expressing CD4$^+$ T cells in HAART-treated patients inversely correlates with immune activation. (A) Flow cytometric analysis of CD25- and FoxP3-expressing CD4$^+$ T cells from HAART-treated, ES, and uninfected patients. Representative FACS profiles of two patients from each group are shown. (B) CD4$^+$ T cells expressing the FoxP3 protein were depleted for patients on HAART compared to levels for ES and uninfected patients ($P < 0.01$). HLA-DR expression on CD4$^+$ T cells was significantly increased for patients on HAART over levels for ES and uninfected patients (top graph). No significant differences in coexpression of FoxP3 and CD69, HLA-DR, or Ki67 were observed (bottom graph). At least four different patients from each group were analyzed. (C) Correlation between FOXP3 copy number and HLA-DR expression on CD4$^+$ T cells from HIV-infected donors ($P < 0.05$).

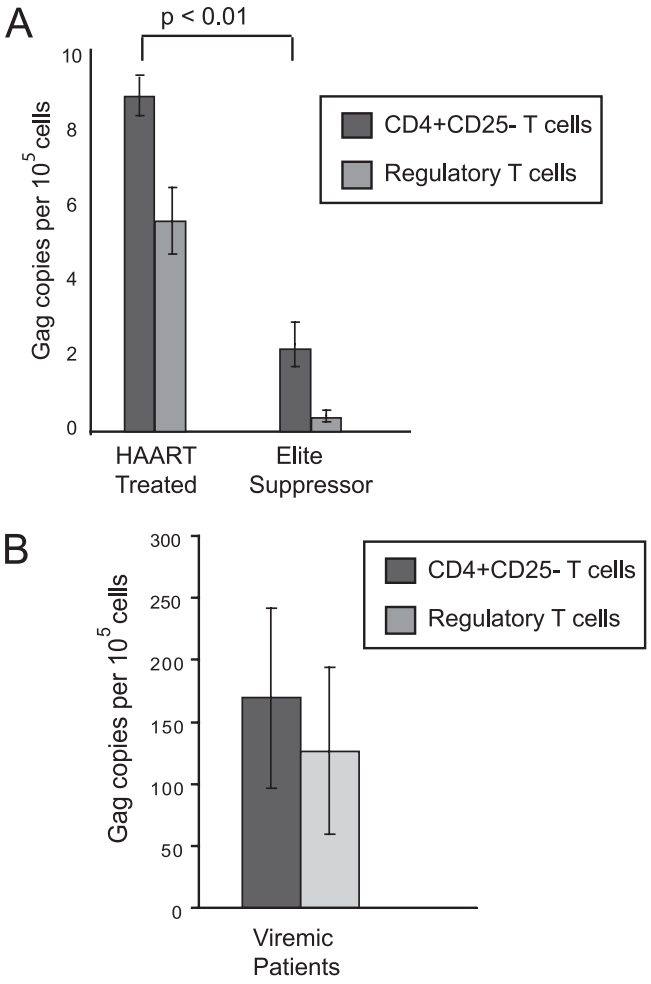

FIG. 3. Peripheral blood Tregs are infected in vivo. Cell-associated viral loads for sorted $CD4^+$ $FoxP3^+$ $CD25^+$ and $CD4^+$ $FoxP3^-$ $CD25^-$ T cells. The average viral load for the two subsets is shown in the bar chart. (A) At least three different HAART-treated and ES patients are analyzed. (B) Representative graph of viral load in Tregs versus that in $CD4^+$ $CD25^-$ resting cells is shown for viremic patients.

difference in the infection rate approached statistical significance ($P = 0.07$) (Fig. 3). The infection frequency of $CD4^+$ $FoxP3^-$ $CD25^-$ resting T cells from the peripheral blood of ES was significantly lower than that observed for patients on HAART ($P < 0.01$). In viremic individuals, Tregs were infected at a rate of 126.4 to 760.5 per 10^5 while resting $CD4^+$ T cells were infected at a frequency of 169.2 to 604.3 per 10^5. Overall, the results show that $CD4^+$ $FoxP3^+$ $CD25^+$ Tregs are infected by HIV-1 in vivo but not at a higher frequency than $CD4^+$ $FoxP3^-$ $CD25^-$ resting T cells (Fig. 3). Thus, the depletion of Tregs from the peripheral blood of patients with progressive disease may be associated with relocation to tissue sites and/or deficiencies in their maintenance rather than preferential infection.

Freshly isolated Tregs from aviremic HIV-1-infected patients were stimulated in vitro to detect cells harboring replication-competent HIV-1. Culture supernatants consistently tested negative for virus production. While we were able to culture replication-competent virus from Tregs from viremic patients, it is not clear whether the replication-competent virus was derived from integrated proviruses or unintegrated viruses from cells that had recently been infected. The lack of virus production from Tregs in HAART-treated patients could be due to a block in viral production after integration that is specific to Tregs whereby the observed viral production from Tregs in viremic patients would be coming from recently infected cells harboring unintegrated virus (25).

Treg functional activity in HAART-treated and ES patients. In order to measure the functional activity of Tregs, it was necessary to isolate a pure population from PBMCs. The only known surface characteristics that distinguish Tregs from activated cells are the levels of CD25 expression and CD62L (L-selectin) (8, 9, 13, 18). In order to rule out contamination with activated/effector cells in our assays, we used expression of CD62L to sort for Tregs, because CD62L is down-regulated upon lymphocyte activation. Discrimination of Tregs from activated cells was achieved by sorting for the $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ population (Fig. 4). Mean FoxP3 expression by flow

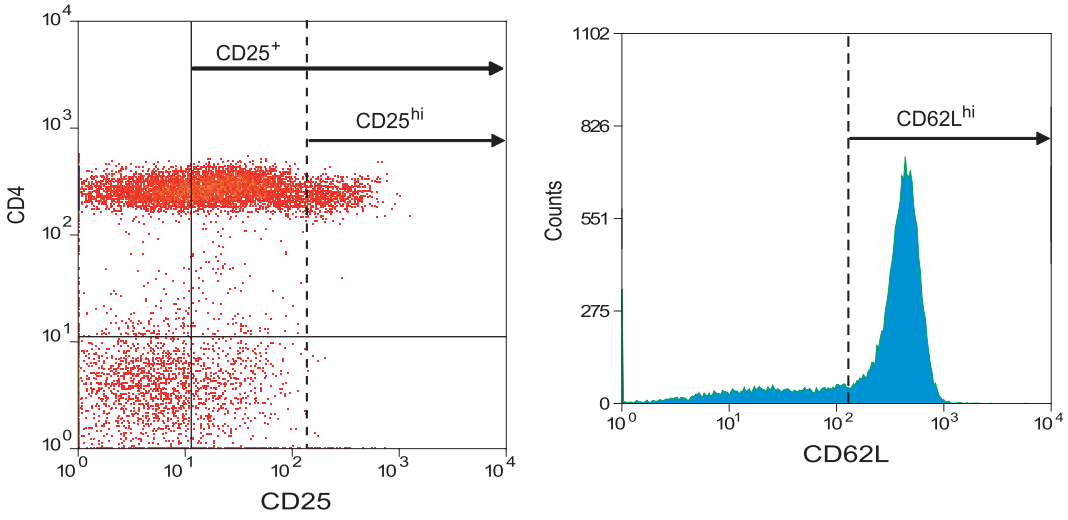

FIG. 4. Identification of Tregs. Freshly isolated PBMCs from HIV-positive patients were first negatively selected and then stained with anti-CD62L fluorescein isothiocyanate, anti-CD25 phycoerythrin, and anti-CD4 tricolor. After gating on $CD4^+$ T cells, Tregs were defined as $CD25^{hi}$ and $CD62L^{hi}$.

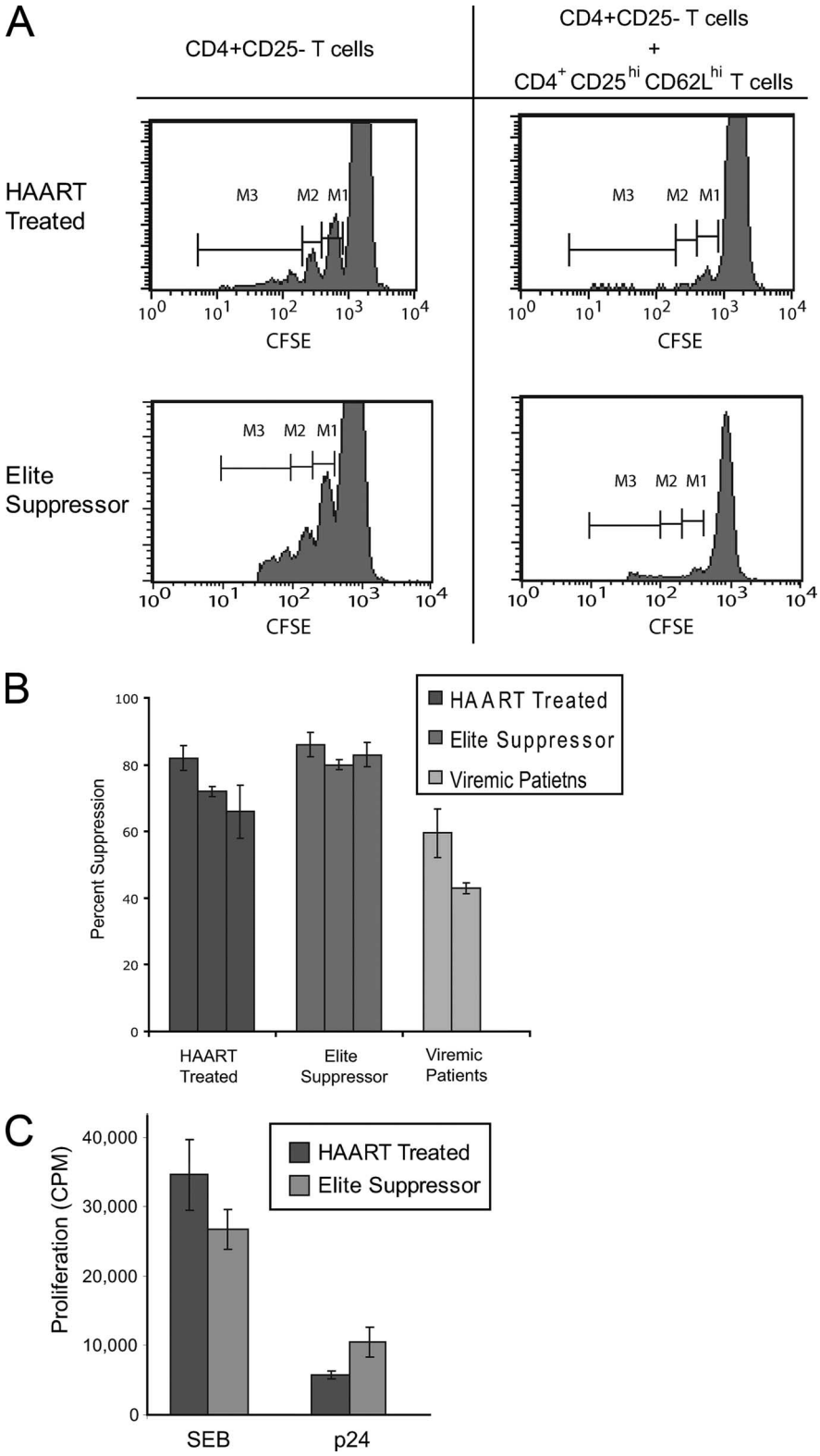

FIG. 5. Treg functional activities are comparable for HAART-treated and ES patients. (A) $CD4^+$ $CD25^-$ $CD62L^{hi}$ naïve T cells from either HAART-treated or ES patients were labeled with CFSE and cultured with anti-CD3 stimulation and irradiated autologous PBMCs. To measure suppressive activity, $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs were added at day zero. Dilution of CFSE was measured by flow cytometry at day 6 poststimulation. Representative FACS profiles from each patient group are shown. (B) Summary of the percent suppression of proliferation of $CD4^+$ $CD25^-CD62L^{hi}$ naive T cells by $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs for three different HAART-treated or ES patients and two viremic patients is provided in graph form. All suppression assays were set up in triplicate, and results are expressed as means of triplicates. (C) Proliferative responses of $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs to cytokine signals are comparable for HAART-treated and ES patients. Sort-purified $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs were maintained in transwell cocultures for 6 days with anti-CD3- and anti-CD28-stimulated autologous PBMCs in the bottom wells. Proliferative responses induced by either SEB or p24 were compared. All proliferation assays were set up in triplicate, and at least three different HAART-treated and ES patients were analyzed.

cytometric analysis of sorted cells was 88% (range = 82 to 93). As determined by real-time RT-PCR, there was an 8.9-fold ± 1.4-fold increase in FoxP3 expression in $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs versus expression in $CD4^+$ $CD25^-$ $CD62L^{hi}$ naive T cells. The gated population was enriched in its expression of CD45RO, CTLA-4, and GITR (data not shown), consistent with the known immunophenotype of Tregs (18, 44, 46).

Tregs suppress the activation and proliferation of effector cells. To determine whether the decrease in frequency of peripheral blood $FoxP3^+$ Tregs in patients on HAART was accompanied by a decrease in the functionality of these cells, we compared the suppressive capacity of Tregs from ES, viremic individuals, and patients on HAART. As shown in Fig. 5A, the $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Treg subset in all patient groups limited division of naive cells stimulated by TCR cross-linking. There was no difference in the suppressive function of Tregs in ES and patients on HAART, but the suppressive activity of Tregs in viremic individuals appeared to be somewhat diminished compared to that in both groups (Fig. 5B).

Another way to address Treg functional differences between patient groups is to measure Treg proliferative capacity. IL-2 is essential to the development and survival of Tregs (3, 17, 37). With the provision of exogenous IL-2, Tregs can proliferate to antigen-loaded APCs or to anti-CD3-based stimulation (51, 53). A transwell culture system was used to analyze cytokine-induced Treg proliferation in response to autologous PBMCs that were stimulated with either SEB or p24. There were no significant differences in the proliferative capacity of peripheral blood Tregs of HAART-treated and ES patients (Fig. 5C). Proliferation of freshly isolated $CD4^+$ $CD25^{hi}$ $CD62L^{hi}$ Tregs without stimulation was at background levels (data not shown). Thus, analysis of Treg suppressive and proliferative activity in HAART-treated and ES patients suggested that there were no functional differences in their peripheral blood Treg populations. Increased $CD4^+$ T-cell activation in patients on HAART was manifested not by a decrease in Treg suppressive activity but rather by a decrease in the number of peripheral blood Tregs that are available to control immune activation.

DISCUSSION

Tregs are thought to play a major role in the control T-cell self-reactivity and immune activation. Because of the common mechanisms involved in immune activation associated with autoimmunity and microbial exposure, Tregs can cause down-modulation of pathogen-specific immune responses. Several groups have postulated that Tregs help to limit the chronic immune activation associated with HIV-1 infection and are therefore beneficial to the host (6, 8, 15, 18, 32, 41, 44, 54). Yet other groups argue that the suppression of HIV-1-specific effector cells can be detrimental to the host (1, 5, 11, 21, 31, 36, 42, 56). It remains to be determined what plays a greater role in HIV-1 pathogenesis, the advantageous effects of controlling immune activation or the harmful effects caused by the limitation of an antiviral immune response. We analyzed the frequency of Tregs in ES in an attempt to discern whether these cells play a role in the nonprogressive nature of the disease in this group of patients.

We used both quantitative RT-PCR and flow cytometric analysis to show that peripheral blood Tregs are preserved in ES, in contrast to their decline in patients with progressive disease, including patients on HAART. There was a negative correlation between the frequency of Tregs and the percentage of activated $CD4^+$ T cells, suggesting that in patients in which the peripheral Treg pool is preserved, a mechanism exists to limit immune activation. In both HAART-treated and ES patients, the frequency of infection within the $FoxP3^+$ Treg subset was lower than the frequency of infection in resting $CD4^+$ T cells.

Some (11, 31, 42) but not all (40) prior studies have shown that Tregs migrate to lymphoid tissue in HIV-1-infected chronic progressors but not in LTNP. The observation that Tregs do not relocate to lymphoid tissue in LTNP may explain the preservation of peripheral Tregs in ES in our study. The maintanence of a peripheral Treg pool in ES could be a consequence of low levels of viral replication at lymphoid sites. A small study showed that viral loads in the intestinal mucosa of LTNP are undetectable (50); thus, Tregs may not home to this site in these patients. Further studies will be needed to determine the relative frequency of Tregs in the lymphoid tissue of ES. Our functional studies suggested that Tregs from patients on HAART retain the same degree of functional activity as Tregs from ES patients. The reported lack of tissue relocation in LTNP may suggest that accumulation of Tregs at tissue sites contributes to HIV-1 pathogenesis (5, 11, 20–21, 31, 42). Alternatively, it may be a reflection of the control of viral replication at these sites (50).

In summary, our observations indicate that peripheral Treg depletion in HIV-1 infection may be a contributing factor to the high levels of immune activation present in patients with progressive disease, since Treg depletion was not observed in ES. Tregs are not preferentially infected; thus, the question remains whether their depletion in patients with progressive disease is due to death by infection or redistribution to lymphoid tissue. Our studies emphasize that there is a delicate balance between the qualitatively superior HIV-1-specific immune response that inhibits replication of HIV-1 in ES (2, 4, 10, 19, 39, 47) and the regulatory mechanisms that prevent nonspecific immune activation in these patients. Successful therapeutic vaccines will have to induce this balance in patients with progressive HIV-1 disease.

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